

## STATE OF THE ART: CONCISE REVIEW

# Biomarkers in Early-Stage Non-Small-Cell Lung Cancer

## Current Concepts and Future Directions

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**Abstract:** Advances in molecular biology and bioinformatics have resulted in the identification of a number of potential biomarkers that could be relevant in the management of patients with non-small-cell lung cancer (NSCLC). Although there is an increasing amount of literature related to these biomarkers, major issues need to be resolved including validity and reproducibility of results. Additionally, in order to interpret the existing literature accurately, a clear distinction must be made between the prognostic and predictive value of biomarkers. The practical applicability of biomarker discovery for patients with lung cancer includes the identification of patients with early-stage NSCLC who are most likely to benefit from adjuvant therapy. Information gleaned from biomarkers has the potential to help in evaluating the role of targeted therapies including immunotherapy in the neoadjuvant and adjuvant setting. The role of gene signatures and the use of newer platforms such as RNA, methylation, and protein signatures is being explored in patients with early-stage NSCLC. This review focuses on the applications of biomarker discovery in patients with early-stage NSCLC.

**Key Words:** Lung cancer, Biomarkers, Early-stage lung cancer, Predictive, Prognostic.

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Lung cancer is the leading cause of cancer-related mortality in the United States and the worldwide. Non-small-cell lung cancer (NSCLC) is the most common form of lung cancer. Early-stage NSCLC (ES-NSCLC; stages I and II) accounts for approximately 18% of the cases.<sup>1</sup> Most of these patients are treated with curative intent and often require multimodality therapy.<sup>2,3</sup> Despite these aggressive measures, the survival associated with ES-NSCLC is less than optimal with a 5-year overall survival (OS) ranging from 50% for stage IA disease to 15% for stage IIIA NSCLC.<sup>4</sup>

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ES-NSCLC has assumed particular significance in recent years for two main reasons. First, the incidence of ES-NSCLC is expected to rise due to the use of computed tomography screening of high-risk patients which has demonstrated a survival benefit.<sup>5</sup> Second, the outcomes of ES-NSCLC may potentially benefit from an improved understanding of the molecular and immunologic basis of NSCLC which has already led to improved outcomes in advanced NSCLC.

Several clinical trials have demonstrated improved survival with postoperative chemotherapy in selected patients who undergo complete surgical resection.<sup>6,7</sup> Available evidence supports the use of adjuvant chemotherapy for stage II and stage IIIA, but not for stage IA NSCLC.<sup>8</sup>

There are several shortfalls to the current approach of selecting patients for adjuvant therapy based on the surgical stage alone. Given the marginal benefits and potential toxicities associated with chemotherapy, perhaps the greatest challenge lies in the identification of patients at the greatest risk of recurrence. One approach to identifying high-risk patients focuses on the biology of ES-NSCLC in an effort to predict the risk of recurrence and the potential for response to treatment by using biomarkers.

A biomarker is a “characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.” A prognostic biomarker is a factor that is associated with an outcome that is independent of treatment, whereas a predictive biomarker interacts with the treatment to influence outcome.<sup>9</sup> There is good clinical evidence for a limited number of biomarkers that are used in clinical practice. Examples include the use of hormone receptor status in breast cancer. These biomarkers are prognostic of improved survival independent of cancer treatment and also predict the benefit of hormonal therapy with drugs such as tamoxifen.<sup>10</sup> Despite a concerted effort, there is a lack of biomarkers with potential application in the management of ES-NSCLC.

The search for a prognostic and predictive biomarker has to take into consideration two key points: the strength of evidence to support its use and the depth of information provided by a biomarker that adds to what is already known about the disease based on the clinical parameters. Although a plethora of potential prognostic biomarkers have been proposed in the past couple of decades, very few have been validated. In this review, we have focused on a very small number of these biomarkers, including immune markers and molecular signatures relevant to ES-NSCLC because of the potential promise associated with them.

## PROGNOSTIC BIOMARKERS

### P53

The tumor suppressor gene, *p53* is frequently altered in NSCLC.<sup>11</sup> Although it is a well-established poor prognostic factor in many tumors,<sup>12,13</sup> in ES-NSCLC its prognostic role is controversial. A subgroup analysis of *CALGB 9633*, a phase III trial that randomized patients with stage IB NSCLC to observation or adjuvant chemotherapy, showed that *p53* expression by immunohistochemistry (IHC) was detectable in 47% of the tumors, and correlated with shorter disease-free survival (hazard ratio [HR], 1.95;  $p = 0.003$ ) and OS (HR, 2.30;  $p = 0.0005$ ) in multivariate analyses.<sup>14</sup> A meta-analysis of pooled patient data from 43 studies which included patients with ES-NSCLC who underwent potentially curative resection showed that *p53* mutation or overexpression was an indicator of poor prognosis, especially in patients with adenocarcinoma (ADC). Compared with patients with no alterations, patients with ADC and *p53* overexpression or mutations had a 21.8% ( $p = 0.000039$ ) and 48% ( $p = 0.000031$ ) reduction in 5-year OS, respectively.<sup>15</sup>

### KRAS

RAS belongs to the family of small guanosine triphosphatase (GTPase) proteins. Rodenhuis et al. first reported an association between *KRAS* mutations and NSCLC. They studied 39 NSCLC samples for the presence of *NRAS*, *KRAS*, and *HRAS* mutations or amplifications and concluded that mutational *KRAS* activation may be an important early event in the pathogenesis of ADC of the lung.<sup>16</sup> They also showed that *KRAS* mutations were present in more than 30% of the ADCs and was more frequent in smokers.<sup>17</sup> Studies in ES-NSCLC report that *KRAS* mutations, especially at codon 12, are associated with worse progression-free survival (PFS) and OS.<sup>18,19</sup> Slebos et al.<sup>20</sup> were the first to show that differences in PFS and OS in patients with ES-NSCLC with and without *KRAS* mutations were significant ( $p = 0.038$  and  $p = 0.001$ ). The prognostic significance of *KRAS* in NSCLC was evaluated in a combined analysis of eight studies with a total of 881 patients. *KRAS* mutations were detected in 25% of the cases and involved codons 12, 13, and 61 of the *KRAS* gene. For the *KRAS* mutant group, the relative risk for mortality was 2.35 (95% CI, 1.61–3.22), compared with patients with wild type *KRAS*. However, these studies were heterogeneous and there were no adjustments for other clinical variables.<sup>21</sup> In recent studies, the relevance of different amino acid substitutions in *KRAS* has been analyzed. Preclinical and retrospective data point out the importance of specific *KRAS* mutations on the prognosis of NSCLC, such as G12C or G12V in contrast to other substitutions.<sup>22</sup> Despite the data presented above, the prognostic significance of *KRAS* remains controversial. In an analysis involving 300 patients with ES-NSCLC with tumors harboring *KRAS* mutations enrolled in four adjuvant trials, the presence or absence of mutations in *KRAS* codon 12 did not confer a survival disadvantage in the observation arms of these trials.<sup>23</sup>

## PROGNOSTIC AND PREDICTIVE BIOMARKERS

### Epidermal Growth Factor Receptor

The epidermal growth factor receptor (EGFR) is a member of the tyrosine kinase cell surface receptor family. Mutations in the gene encoding this protein results in constitutive activation and amplification of intracellular signals which lead to proliferation, invasion, and migration of the cancer cells.<sup>24</sup> The overall implications of the presence of *EGFR* mutations or amplification in ES-NSCLC are not well defined. Rusch et al.<sup>25</sup> detected *EGFR* overexpression by IHC in 74 (71%) of 96 ES-NSCLC tumor samples. However, there was no association with OS. In a separate study, 53 ES-NSCLC tumor samples (79% ADC) were analyzed for the presence of *EGFR* mutations in exon 19 and 21 by polymerase chain reaction (PCR), and 32% samples were found to harbor mutations. Presence of an *EGFR* mutation was identified as a favorable prognostic factor, with 5-year OS of 92% for *EGFR*-mutated versus 57% for *EGFR* wild-type tumors ( $p = 0.037$ ).<sup>26</sup> The same group reported a retrospective analysis of 180 patients with either *KRAS* codon 12 mutation or *EGFR* mutation (exons 18–21). This study showed that the presence of an *EGFR* mutation was associated with longer OS ( $p = 0.048$ ). However, there was no impact on PFS.<sup>27</sup> Liu et al.<sup>28</sup> examined 130 ES-ADC samples for *EGFR* mutations in exon 19 and 21 by nested PCR, and detected mutations in 44.3% samples. Presence of an *EGFR* mutation did not have an impact on median PFS (36.6 months for *EGFR*-mutated versus 25.7 months for *EGFR* wild-type tumors;  $p = 0.56$ ). A large retrospective study reported the outcome of 1118 patients with resected ES-NSCLC of whom 20% had an *EGFR* mutation. The presence of an *EGFR* mutation correlated with longer OS (HR, 0.51;  $p < 0.001$ ). A subgroup analysis was conducted in from a different dataset of 286 resected ES-NSCLC ADCs harboring *EGFR* mutations to determine the effect of adjuvant *EGFR* tyrosine kinase inhibitor (TKI) therapy. Among 286 patients receiving adjuvant TKI Cox regression analysis demonstrated a significant improvement in PFS (HR, 0.43;  $p = 0.001$ ), but no significant differences in OS.<sup>29</sup>

Although, as illustrated above multiple retrospective analyses demonstrate improved survival in patients with completely resected *EGFR*-mutated ES-NSCLC, definitive conclusions can only be drawn by conducting large prospective clinical trials in this patient population.

### Her2

*Her2*, a receptor tyrosine kinase and a member of the *EGFR* family is overexpressed in 20% of the advanced NSCLC and mutated in less than 2%.<sup>30,31</sup> In ES-NSCLC, studies suggest that overexpression of *Her2* mRNA or protein is associated with an unfavorable prognosis.<sup>32,33</sup> In a retrospective study, 239 tumor samples of patients with ES-NSCLC were evaluated for *Her2* overexpression, which was defined as an IHC score of 2+/3+ (scoring based on staining intensity and the number of cells stained). *Her2* overexpression was detected in 18% of the tumors. The relapse rate for *Her2*-positive versus *Her2*-negative tumors was 60% versus 33%, respectively ( $p = 0.03$ ) in the absence of adjuvant treatment.<sup>34</sup>

A different definition of *Her2* positivity by IHC was used by Xia et al. who considered *Her2* overexpression as any positive staining. In a study involving only stage I and IIA NSCLC, 74% of ADCs, and 54% of the squamous cell carcinoma (SCC) were found to overexpress *Her2*. The 5-year OS was 65% versus 86% in *Her2*-positive versus *Her2*-negative tumors respectively ( $p = 0.014$ ).<sup>35</sup> Despite differences in the criteria used to define *Her2* overexpression, both studies attribute negative prognostic value to *Her2* overexpression in ES-NSCLC. Although the presence of a *Her2* mutation can predict for the response to *Her2*-directed therapy in the metastatic disease setting, its predictive value remains unclear in ES-NSCLC.<sup>36</sup>

### Excision Repair Cross-Complementation Group 1

The excision repair cross-complementation group 1 (ERCC1) protein plays a rate-limiting role in the nucleotide excision repair pathway that recognizes and removes cisplatin-induced DNA adducts.<sup>37</sup> In ES-NSCLC, tumor *ERCC1* expression appears to have both prognostic and predictive value. In one of the first studies evaluating *ERCC1*, 51 resected samples of NSCLC were analyzed for *ERCC1* RNA expression by PCR. Overexpression was associated with improved survival (HR, 0.337;  $p = 0.018$ ), suggesting the value of ERCC1 expression as a prognostic biomarker.<sup>38</sup> Subsequent studies focused on the role of this gene as a predictive marker, wherein low *ERCC1* expression levels are predictive of the benefit from cisplatin-based adjuvant chemotherapy. ERCC1 expression by IHC was studied in patients with stage I–III NSCLC enrolled in the International Adjuvant Lung Cancer Trial. Adjuvant chemotherapy prolonged survival among patients with ERCC1-negative tumors (HR, 0.65;  $p = 0.002$ ), but not among patients with ERCC1-positive tumors (HR, 1.14;  $p = 0.40$ ). However, subjects with ERCC1-positive tumors had better survival than subjects with ERCC1-negative tumors in the observation arm (HR, 0.66;  $p = 0.009$ ). This and other studies have demonstrated the prognostic and predictive value of ERCC1 in ES-NSCLC.<sup>39,40</sup>

Despite these interesting results, utilization of ERCC1 as a predictive marker is limited by the lack of reproducibility of results with the antibodies used in the IHC assay. Friboulet et al. repeated ERCC1 IHC staining on the original testing set of 589 samples from the International Adjuvant Lung Cancer Trial, and attempted to unsuccessfully validate the results utilizing 494 samples collected from two independent phase III trials, JBR10 and CALGB 9633. There was a lack of correlation between the 16 different antibodies used, and an inability of these antibodies to identify various ERCC1 isoform specificities.<sup>41</sup>

### Ribonucleotide Reductase M1

Ribonucleotide reductase M1 (RRM1) is the regulatory protein subunit of ribonucleotide reductase and is involved in DNA repair, carcinogenesis, and cancer progression. The mechanistic relationship between RRM1 levels and sensitivity of tumors cells to gemcitabine has been demonstrated in preclinical studies.<sup>42</sup> In ES-NSCLC, several studies have

suggested a prognostic role for RRM1. In a study of 126 patients with ES-NSCLC *RRM1* expression was evaluated by PCR analysis. This study demonstrated better median OS in patients with higher levels of *RRM1* (52 months versus 24 months,  $p = 0.013$ ).<sup>43</sup> Similarly, RRM1 expression by automated quantitative protein analysis was studied in 187 patients with stage I NSCLC. In this study, the median OS was 120 months versus 60.2 months in the group with high RRM1 expression compared with the low expression group ( $p = 0.01$ ).<sup>40</sup> To evaluate the predictive potential of RRM1 expression in the context of treatment with gemcitabine, a phase II study was conducted in the ES-NSCLC population with the choice of adjuvant therapy determined by RRM1 protein expression. Eighty-five patients with stage I NSCLC were enrolled and the treatment was customized based on the expression of *ERCC1* and *RRM1*. Among patients with RRM1-expressing tumors, the PFS at 2 years was 83% in patients receiving gemcitabine compared with 71% in patients in the observation arm. No statistical tests were performed to evaluate the significance of these observations. Hence, further studies are needed to determine the predictive value of *RRM1* expression in ES-NSCLC.<sup>44</sup>

### BRCA

The *BRCA* gene is a tumor suppressor involved in the homologous recombination repair pathway, a mechanism for DNA repair. Methylation or mutations which decrease *BRCA* expression or activity have been shown to cause impairment in the homologous recombination repair pathway. The value of *BRCA1* and *BRCA2* germ line mutations and the risk of developing breast and ovarian cancer are well established.<sup>45</sup>

Expression of BRCA protein in ES-NSCLC was assessed by IHC in 98 tumor samples. Among 50 cases of ES-NSCLC, 50% samples showed low levels of *BRCA1* and *BRCA2* protein.<sup>46</sup> Marsit et al. described the methylation status of *BRCA* genes and its relationship to prognosis in 158 ES-NSCLC patients. Only 3.8% of the tumors were methylated (only ADC and large-cell carcinomas). No methylation in *BRCA2* was detected and the methylation of *BRCA1* was associated with worse OS (HR 3.1; 95% CI, 1.2–7.9).<sup>47</sup> Rosell et al. examined nine genes involved in the nucleotide excision repair pathway, including *BRCA1* in 126 ES-NSCLC patients, none of whom had received adjuvant chemotherapy. Among the genes studied, only high *BRCA1* mRNA expression correlated significantly with worse OS (HR, 1.98;  $p = 0.02$ ).<sup>48</sup> In the adjuvant setting, the predictive value of *BRCA1* was studied in 83 patients with ES-NSCLC (stage II–IIIA) and the treatment was tailored according to *BRCA1* mRNA levels. With a median follow-up of 41.6 months, the median time to progression for the all patients was 22.9 months. Based on *BRCA1* levels, the median time to progression was 20.3, 56.5, and 51.9 months for the low, intermediate, and high level groups respectively ( $p = 0.31$ ) and the differences were not statistically significant.<sup>49</sup> Despite its promise as a potential predictive biomarker of platinum efficacy, there is a lack of evidence to define *BRCA* as a clinically relevant biomarker in ES-NSCLC.

Table 1 summarizes the biomarkers evaluated in ES-NSCLC.



**TABLE 1.** Summary of Biomarkers Evaluated in ES-NSCLC

Gene	Protein Function	Author and Year	Method	Number of Patients	Comments
<i>TP53</i>	Involved in growth arrest and apoptosis	*Mitsudomi <sup>15</sup> , 2000	IHC	2579	Prognostic for survival
			Gene sequencing	650	
<i>KRAS</i>	Transduction protein with GTPase activity	*Shepherd <sup>23</sup> , 2013	PCR	1543	Not of significant prognostic or predictive value
<i>EGFR</i>	Tyrosine kinase cell surface receptor	Liu <sup>28</sup> , 2014	PCR	131	Not significantly prognostic
<i>ERBB2</i>	Tyrosine kinase cell surface receptor	Xia <sup>35</sup> , 2012	IHC	172	Prognostic for survival
<i>ERCC1</i>	Enzyme involved in DNA damage repair	Friboulet <sup>41</sup> , 2013	IHC	589	Not predictive for response to chemotherapy
<i>RRM1</i>	Enzyme essential for the production of deoxyribonucleotides	Zheng <sup>40</sup> , 2007	AQUA	187	Prognostic for survival
<i>BRCA</i>	Participate in the repair of DSB	Rosell <sup>48</sup> , 2007	PCR	126	Prognostic for survival

PCR, polymerase chain reaction; IHC, immunohistochemistry; AQUA, absolute quantification; DSB, double strand break; ES-NSCLC, early-stage non-small-cell lung cancer.  
 \*represents meta-analysis.

## IMMUNE SYSTEM AND ES-NSCLC

Immunotherapeutic approaches are beginning to play an increasingly important role in the treatment of solid tumors. There have been important advances in this field leading to the approval of ipilimumab in melanoma,<sup>50</sup> sipuleucel-T vaccine in prostate cancer,<sup>51</sup> and reports of clinical activity of monoclonal antibodies (mAbs) against programmed cell death-1 (PD-1) and its ligand (PDL-1) in solid tumors.<sup>52</sup>

Past trials of immunotherapy in lung cancer have demonstrated a limited activity. Immune mechanisms proposed to explain the lack of success in lung cancer include underexpression of the classic major histocompatibility complex molecules, overexpression of soluble factors such as prostaglandin E2 (PGE2) and transforming growth factor beta (TGF- $\beta$ ) and overexpression of specific enzymes such as COX-2 and Ido-1+, which help in circumventing successful immune control.<sup>53</sup> More recently, checkpoint inhibition with anti-PD1 and anti-PDL1 therapies has yielded remarkable results in heavily pretreated patients with NSCLC and provided an impetus to further explore the role of immunotherapy in the treatment of lung cancer.

## TUMOR INFILTRATING LYMPHOCYTES AS PROGNOSTIC BIOMARKERS

Previous studies have demonstrated the prognostic significance of tumor infiltrating immune cells and the location and density of these infiltrates in ES-NSCLC.<sup>54</sup> In a retrospective study of 1290 patients with lung cancer a correlation was shown between tumor infiltrating lymphocytes (TILs), and prognosis in a subgroup of patients with stage I SCC. Presence of TILs was associated with a significant survival advantage over tumors without TILs ( $p = 0.03$ ).<sup>55</sup>

Among studies that evaluated specific T-cell subsets and their localization in ES-NSCLC, Wakabayashi et al.<sup>56</sup> studied 178 ES-NSCLC and showed that the location of CD8 (+) T cells (nests compared with stromal areas) had an effect on the prognosis. The actuarial 5-year OS was 47% versus 60% ( $p = 0.04$ ). Similarly, the proportion of TILs (CD4 (+), CD8(+), CD20(+)) in areas enriched for tumor epithelial cells compared with tumor stroma was examined in 335 cases of ES-NSCLC. It was observed that the presence of

larger numbers of stromal CD4(+) and CD8(+) T-cells was an independent prognostic factor for longer disease-specific survival compared with epithelial infiltration with HRs of 2.6 ( $p < 0.001$ ) and 1.98 ( $p < 0.043$ ), respectively.<sup>57</sup> These observations merit further study to determine the significance of T-cell infiltration of the stroma.

Regulatory T cells (Tregs or Foxp3-positive T cells) are a specific subset of the T cell repertoire which have been demonstrated to be involved in decreasing the antitumor response.<sup>58</sup> It has been shown that recurrence-free survival (RFS) of the patients with tumors containing 3 or greater Tregs in 10 high-power fields was significantly worse than that of patients with tumors containing < 3 Tregs/high-power field, especially in stage I and II disease (HR, 5.38;  $p < 0.016$ ).<sup>59</sup> A recent study has shown the prognostic significance of the ratio of Tregs to CD3 compared with Tregs alone in 956 cases of stage I NSCLC. A low Tregs to CD3 ratio was found to be associated with a longer RFS (5-year RFS: 85% versus 77%;  $p = 0.004$ ).<sup>60</sup> Of note, the better-than-expected 5-year survival observed in both the groups is probably a result of the selection bias since a large fraction of the patients enrolled on this study had favorable prognostic factors including the absence of lymphatic and vascular invasion.

## CHECKPOINT MOLECULES AS PROGNOSTIC AND PREDICTIVE IMMUNE BIOMARKERS

A relatively new area of research in cancer treatment focuses on the role of regulatory signals through molecules like cytotoxic T-lymphocyte antigen 4 (CTLA-4) and PDL-1. CTLA-4 is a well-known inhibitory molecule of T cell activation expressed on Tregs. Its expression occurs in the context of the interaction between T-cell receptors and antigen presentation. The expression of CTLA-4 on Tregs can provide an explanation for the prognostic significance of Tregs infiltration in ES-NSCLC as previously described.<sup>59,61</sup> CTLA-4 overexpression has been found to be more common in ADC compared with SCC and appears to be an independent favorable prognostic factor (5-year OS of patients with CTLA-4-overexpressing tumors: 64.8% versus 45.9%;  $p = 0.078$ ).<sup>62</sup>

PD-1 is a checkpoint receptor that has immunosuppressive functions similar to CTLA-4.<sup>63</sup> However a few key

differences between these immune checkpoints include the following: (1) PD-1 is activated during the effector stages of T-cell activation, (2) the interaction of PD-1 with its ligand PD-L1 occurs primarily in peripheral tissues instead of lymph nodes, and (3) PD-1 is expressed in tumor tissue as well as hematopoietic cells.<sup>64</sup> A phase I study by Topalian et al. found that 50% of the lung tumors with PD-1 expression (defined as  $\geq 5\%$  expression) responded to anti-PD-1 mAb compared with 0% of PD-1 negative tumors.<sup>52</sup> While the study included patients with advanced disease, these results are encouraging and potentially applicable to ES-NSCLC in the future.<sup>65</sup> It should be noted, however that although associations have been demonstrated between expression of and response to mAbs targeting PD-1 and PD-L1, recent observations also show that the expression of PD-1 and PD-L1 is not a prerequisite for the generation of a therapeutic response.<sup>66,67</sup> This important point needs to be borne in mind as studies are designed in the future incorporating anti-PD-1/PD-L1 therapies for patients with ES-NSCLC.

Studies performed thus far to evaluate the prognostic value of PDL-1 expression in ES-NSCLC have generated discordant results. In one of the largest validation studies for PD-L1 expression (any level of expression in tumor cells above the negative control was considered a positive result), Velcheti et al., demonstrated PD-L1 protein expression in two separate cohorts (enrolled in two different countries) of NSCLC that included 88% and 84% cases with stage I to III disease respectively. Expression of PDL-1 was detected in 36% and 25% cases in the two cohorts and was associated with better OS independent of others factors including histology in each of the cohorts ( $p = 0.036$  and  $0.027$ ).<sup>68</sup> Of note, this study did not analyze the effects of treatment administered. Therefore, although it provides valuable hypothesis-generating data, this study precludes the classification of PD-L1 expression as an established prognostic factor in ES-NSCLC.

## GENE SIGNATURES AS BIOMARKERS

A gene signature is a biomarker in which the expression of multiple genes, proteins, or microRNA (miRNA) is measured and combined into a group with possibly superior

prognostic or predictive potential as compared with its individual components. Data from gene signatures may be presented in the form of risk scores as is the case with the Oncotype DX assay.<sup>69</sup> Signatures may also be presented as categorical subgroups such as activated B-cell-like or germinal center B-cell like diffuse large B-cell lymphoma.<sup>70</sup>

In ES-NSCLC, several studies have attempted to identify gene expression signatures with prognostic or predictive potential by using diverse platforms and genes. Details of some gene signatures evaluated in ES-NSCLC are presented in Table 2 to illustrate the diversity of genes chosen, types of platforms, and the primary goals used to capture prognostic and/or predictive information. One of the challenges in developing gene signatures is to make them simple and reproducible. Chen et al. identified a prognostic reverse-transcriptase (RT)-PCR-based five-gene signature (including *DUSP6*, *MMD*, *STAT1*, *ERBB3*, and *LCK*) using risk scores based on microarray analyses of 125 ES-NSCLC tumor specimens. A high-risk score was associated with increased risk of death (HR for death 1.92,  $p = 0.03$ ).<sup>71</sup> Lau et al. developed another RT-PCR-based signature utilizing a three gene cluster (*STX1A*, *HIF1A*, and *CCR7*) with prognostic significance. Based on analysis of 147 patients, they were able to stratify stage I ES-NSCLC into two prognostic groups. When poor risk scores were compared to good risk scores, the HR for survival was 5.9 ( $p = 0.0019$ ).<sup>72</sup>

These and other early studies of gene expression signatures using RT-PCR evaluated only a limited number of genes as opposed to larger microarray platforms that have tested hundreds of genes with a goal of developing a test that could be easily applied in the clinical setting. These studies are limited by the heterogeneous stages of the disease in the study population, the lack of information on standard clinical predictive factors, and perhaps most importantly, the lack of blinded validation in independent cohorts in most cases. A review of 16 studies published on gene expression profiling in NSCLC from 2002 to 2009 found no evidence of the clinical utility of these signatures beyond that obtained from currently used clinical parameters.<sup>73</sup> More recently, Kratz et al. reported a 14-gene prognostic signature using quantitative PCR in 361 patients with non-squamous ES-NSCLC and validated their

**TABLE 2.** Examples of Studies Evaluating Different Types of Signatures in ES-NSCLC

Signature	Characteristics	Independent Validation	No. of Patients and Stage	Author and Year	Comments
Immune genes signature (meta-analysis)	Different miRNA platforms	Yes	1625* I to III	Suzuki <sup>54</sup> , 2011	Prognostic for PFS
Angiogenic signature	Lung tissue miRNA	No	20 I to III	Donnem <sup>79</sup> , 2012	Prognostic for PFS
DNA methylation signature	450000 CpG sites analyzed	Yes	237 Stage I	Sandoval <sup>76</sup> , 2014	Prognostic for PFS
MicroRNA signature	440 miRNA	Yes	251 I to IIIA	Landi <sup>78</sup> , 2010	Prognostic for OS
Proteomic signature	Mass spectrometry 2630 signals	Yes	116 I to IIIA	Yanagisawa <sup>82</sup> , 2007	Prognostic for OS

N, number, total number of patients from different studies.

miRNA, micro RNA; PFS, progression-free survival; OS, overall survival; ES-NSCLC, early-stage non-small-cell lung cancer.

\* represents meta-analysis.

results in a large cohort of stage I nonsquamous NSCLC. In the validation cohort, the 5-year OS was 71% in low-risk, 58% in intermediate-risk, and 49% in high-risk patients ( $p = 0.0003$ ). Multivariate analysis indicated that the 14-gene signature provided information which added to that obtained from standard staging.<sup>74</sup>

Attempts have been made to develop prognostic gene signatures in ES-NSCLC based on the methylation of DNA bases or acetylation of histones. A study was conducted to determine the methylation status of seven genes in 51 stage I NSCLC patients and 116 matched controls. In multivariate analysis methylation of *p16* and *CDH13* was associated with a shorter time to recurrence (odds ratio for early recurrence 25.25;  $p = 0.006$ ).<sup>75</sup> In a separate study in stage I NSCLC the methylation status of tumors was investigated in 237 cases and validated in a separate cohort of 143 cases. The analysis revealed a five gene methylation signature (*HIST1H4F*, *PCDHGB6*, *NPBWR1*, *ALX1*, *HOXA9*) that could identify patients at a high risk of recurrence (HR, 3.24;  $p = 0.001$ ).<sup>76</sup>

In recent years, the prognostic value of miRNAs, which are small, noncoding, single stranded RNAs that are involved in regulation of gene expression, has been explored in ES-NSCLC.<sup>77</sup> Raponi et al. profiled global miRNA expression of 54 SCC samples and found that high miR-146b expression was associated with a median OS of 26 months versus 95 months, (HR, 2.7;  $p < 0.0035$ ) in the low miR-146b expression group. Landi et al.<sup>78</sup> studied 165 ADC and 125 SCC using a miRNA platform and found that five miRNAs of the let-7 family strongly differentiated ADC from SCC ( $p < 0.0001$ ) and a group of five miRNA signatures correlated with OS, independent of the stage and age ( $p < 0.017$ ).

A number of studies have also been published using different platforms for gene enrichment. This has been done to study clusters in specific pathways, such as immune response or angiogenesis.<sup>54,79</sup> Donnem et al. used this approach to analyze a set of 128 miRNA in a cohort of ES-NSCLC patients. They found a significant correlation between a signature of miRNAs and angiogenic marker fibroblastic growth factor 2 (*FGF2*) ( $r = 0.34$ ;  $p < 0.001$ ). There was no significant correlation with vascular endothelial growth factor A (*VEGF-A*) and hypoxia-inducible factor (*HIF-1 $\alpha$* ).<sup>79</sup> Although miRNAs show promise as a prognostic marker, most of the miRNAs are nonoverlapping in different studies, most likely due to a selection bias and design issues.

Far fewer studies have been published on predictive signatures in lung cancer. Zhu et al. performed a study with a 15-gene expression signature in 133 frozen tumor samples in the JBR.10 adjuvant clinical trial. The signature separated patients without adjuvant treatment into two groups, high-risk and low-risk. The high-risk group had a mortality HR of 15.02 ( $p = 0.001$ ) in both stage I and II compared to the low-risk group. Among 71 patients receiving chemotherapy, the high-risk group was found to have a mortality HR of 0.33 ( $p = 0.005$ ) versus a HR of 3.67 ( $p = 0.013$ ) in the low-risk group.<sup>80</sup> This signature was found to be both prognostic for survival of untreated patients and predictive for survival after adjuvant chemotherapy.

To summarize, several studies have described gene-expression signatures that are associated with the outcome in

**TABLE 3.** Key Points in the Evaluation of Biomarker Studies in ES-NSCLC

Objectives of the trial	Is the objective of the trial to identify prognostic markers, predictive markers or both?
Type of study	Is it a biomarker discovery or validation study or both?
Population	What subgroup of ES-NSCLC does the study include? For example, stage IA or stage IB, which histology squamous vs. nonsquamous. Other subgroups EGFR mutated etc.
Specimen	Is adequate technical information provided regarding tissue processing and storage so that technical bias can be minimized in future validation studies? Is the study performed on easily available samples or on fresh-frozen specimens?
Assay	Is the assay clinically applicable? Is the technique widely available? Was it performed on blinded clinical data?
Validation	Is the biomarker validated in an independent cohort of patients with similar characteristics?
Benefit over standard risk factors	Does the biomarker offer significant benefit or information over standard risk factors? Was independent statistical validation performed?

ES-NSCLC, early stage non–small-cell lung cancer; EGFR, epidermal growth factor receptor.

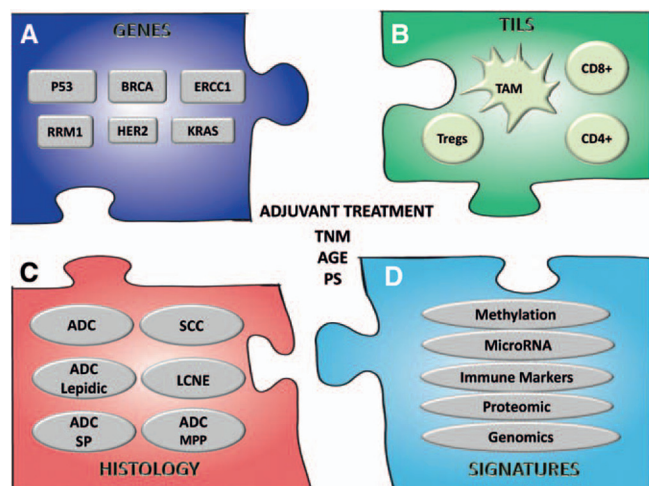
ES-NSCLC. However, most of these studies have one or more caveats; nonprospective validation, inability to provide additional information to that gleaned from standard prognostic factors, and the lack of strong data to alter clinical treatment decisions. Table 3 illustrates key aspects related to the evaluation of prognostic signatures in ES-NSCLC with an emphasis on gene signatures.<sup>81</sup>

## CONCLUSIONS

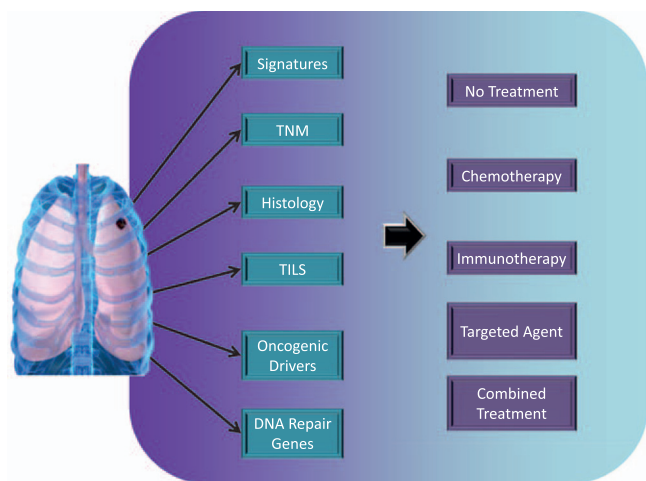
In the present review we have described several potential biomarkers that have been evaluated in patients with ES-NSCLC (Fig. 1). Significant challenges remain in the incorporation of information derived from these biomarkers into the management of patients with ES-NSCLC. A clear distinction has to be made between prognostic and predictive factors especially in relation to the clinical trials designed to evaluate potential biomarkers or when a retrospective analysis is conducted to evaluate a putative biomarker. Figure 2 envisions a future scenario illustrating the use of biomarkers in clinical practice.

Many important issues need to be settled before biomarker-derived information and gene signatures can find widespread acceptance for patients with ES-NSCLC. One important issue is the validity and reproducibility of data. Other aspects that are no less important include reliability in trial implementation and tissue sampling. Uniformity in obtaining and handling biopsy samples is of critical importance. Despite





**FIGURE 1.** Potential biomarkers in ES-NSCLC: (A) single genes; (B) TILs including macrophages (TAM), T lymphocytes CD4(+), CD8(+), and T regulatory cell (Tregs); (C) histology subtypes: ADC, SCC, BRC, LCNE, SP, and MPP; (D) signatures or group of genes, proteins, methylation patterns, microRNAs etc. In the center, prognostic factors used in clinic, staging (TNM), PS, and age. TILs, tumor infiltrating lymphocytes; ADC, adenocarcinoma; SCC, squamous cell carcinoma; BRC, bronchoalveolar carcinoma; LCNE, large cell neuroendocrine tumor; MPP, micropapillary; SP, solid predominant; ES-NSCLC, early-stage non-small-cell lung cancer; TNM, tumor, node, metastasis; PS, performance status.



**FIGURE 2.** Potential future scenarios for using biomarkers in early stage non-small-cell lung cancer. Besides the stage (TNM) and histologic subtype, molecular information from single genes (e.g., oncogenic drivers, DNA repair genes) or groups of genes (signatures) will help in identifying patients who could derive benefit from adjuvant treatment. Immune biomarkers (TILs) could provide prognostic or predictive information relevant to emerging immune therapies. TILs, tumor infiltrating lymphocytes; TNM, tumor, node, metastasis.

these challenges, utilizing technological advances in bioinformatics and genomics has the potential to result in the discovery of newer molecular markers that might have prognostic and predictive value for patients with ES-NSCLC.

Finally, just as in the case of advanced lung cancer where biomarkers have an established role in predicting response to specific TKI therapies, a similar paradigm needs to be explored in ES-NSCLC to identify patients who could derive benefit from neoadjuvant and adjuvant therapy. Immune system-related biomarkers have the potential to represent the next step forward in the effort to develop prognostic and predictive markers for patients with ES-NSCLC.

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